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11/29/01**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Bushby et al. Attorney Docket No.: 109272.130  
Serial No.: 09/487,558 Examiner: K. Davis  
Filed: January 19, 2000 Group Art Unit: 1636  
Entitled: METHODS FOR IMPROVING SECONDARY METABOLITE  
PRODUCTION IN FUNGI  
Assistant Commissioner for Patents  
Washington, D.C. 20231

considered  
LFD  
1-30-02**DECLARATION OF G. TODD MILNE, Ph.D. UNDER 37 C.F.R. § 1.132**

Assistant Commissioner for Patents:

I, G. Todd Milne, declare as follows:

1. My curriculum vitae together with a list of these publications and presentations is enclosed herewith as Exhibit A.

2. I am the Vice President, Research, at the assignee of the patent application, Microbia, Inc. I was a Fellow in the laboratory of Dr. Gerald Fink at the Whitehead Institute for Biomedical Research. I received my Ph.D. in Microbiology and Molecular Genetics from Harvard Medical School. I also hold a B.S. in Molecular Biophysics and Biochemistry from Yale University.

3. I am one of the co-inventors of the above-captioned application.

4. I have read the outstanding Office Action rejections under Section 112, first paragraph. The following data is presented to further prove that the patent application enables the pending claims. The data is presented in seven examples, which are detailed below. These examples are all based on the guidance in the specification as filed and what was well known in the art at the time of the filing.

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## 5. Example 1

### Regulators of lovastatin production in *Aspergillus terreus*

5.1 Purpose. The purpose of this experiment was to examine the effect of expressing fungal regulators on secondary metabolite production in *A. terreus*. Improved production of the antihypercholesterolemic agent lovastatin will aid in the current need for high yielding production processes.

### 5.2 Methods.

In these experiments we have determined whether overexpressing some of these regulators affects lovastatin production in *A. terreus*. Plasmids MB3247 (*ganB*), MB3250 (*gpa3*), MB1310 (*creA*), MB1669 (*LYS14*), and MB2502 (*Pc23*) were transformed into *A. terreus* strain MF22 (ATCC 20542). MB2941 was used as the vector control for MB3247 and MB3250. MB2143 was used as a control for the remainder of the plasmids. The *P. chrysogenum Pc23* gene is a putative regulator of fungal gene expression that encodes a GATA zinc finger transcription factor. Fungal homologs of *Pc23* as well as the G protein  $\alpha$  subunits from *A. nidulans* (*ganB*) and *U. maydis* (*gpa3*) appear in Table 1 of the present specification. *creA* and *LYS14* are both listed in Table 1 of the present specification.

Transformation of *A. terreus* and measurement of lovastatin production were achieved according to the protocols described below. Figure 1, attached to my Declaration, is a graphic depiction of lovastatin culture concentration, as measured by HPLC analysis, from broths of *A. terreus* cultures expressing the regulators. The number of different transformants tested for each plasmid is listed in parentheses next to the label. Results are shown in standard box plot format. The horizontal line in each individual box represents the median. The corresponding vector control is shown in a hatched same colored box.

The results demonstrate that five different regulators from four heterologous species increase lovastatin production in *A. terreus*.

### 5.3 Transformation of filamentous fungi.

Transformation of filamentous fungi was performed according to the following procedure. Protoplasts were generated from spores that were germinated in rich media. Spores were allowed to germinate for about 20 hrs or until germ tubes were between 5 and 10 spore lengths. The germlings were centrifuged and washed twice with sterile distilled water and once with 1M magnesium sulfate. Germlings were then resuspended in 1M magnesium sulfate containing approximately 2mg/ml of Novozyme. Tubes were then incubated at 30°C shaking at 80 rpm for about 2 hours or until most of the hyphae were digested and protoplasts were abundant. Protoplasts were then filtered through one layer of Miracloth. At least one volume of STC was added and protoplasts were centrifuged. Protoplasts were washed twice with STC. Protoplasts then were resuspended in 1ml STC and counted in a hemocytometer. A final concentration of approximately  $5 \times 10^7$  protoplasts/ml was frozen in a 9:1:0.1 solution of STC, SPTC and DMSO in a Nalgene Cryo cooler at -80°C (cools -1°C/min).

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Solutions for transformation were as follows: STC (0.8M Sorbitol, 25mM Tris-HCl pH 7.5, 25mM CaCl<sub>2</sub>) and SPTC (0.8M Sorbitol, 40% PEG 4000, 25mM Tris-HCl pH 8, 50mM CaCl<sub>2</sub>). Transformation was accomplished according to the following protocol. 1-5 µg of DNA comprising the designated plasmid was placed in a 50 ml Falcon tube. 100 µl of previously frozen protoplasts were added to the DNA, gently mixed, and then incubated on ice for 30 minutes. 15 µl of SPTC was added, followed by mixing (by tapping) and incubation at RT for 15 minutes. 500 µl SPTC was added and mixed well by tapping and rolling, then incubated at RT for 15 minutes. 25 mls of regeneration minimal medium was added, mixed well and poured on plates containing 25 mls of regeneration minimal medium with 2X the concentration of selection drug.

Transformation plates were incubated at 26°C for 5-6 days or until colonies started to appear. Regeneration minimal medium contains trace elements, salts, 25mM sodium nitrate, 0.8M sucrose, and 1% agarose at pH 6.5. The selection drug that was used successfully with *A. terreus* is phleomycin, a broad-spectrum glycopeptide antibiotic. Transformants were picked onto new plates with a toothpick (if fungus was sporulating) or with sterile forceps (if fungus did not sporulate). Purification plates contained minimal medium (same as regeneration minimal medium but containing 2% instead of 0.8M sucrose) and 1X drug concentration. Picked transformants were incubated at 26°C for 5-6 days.

Transformants were grown in production media to assess secondary metabolite production. Briefly, for *A. terreus* and lovastatin production, spores were used as the inoculum. Spores were obtained from the purification plate by using a wooden inoculation stick. The medium was RPM containing corn steep liquor, sodium nitrate, potassium phosphate, magnesium sulfate, sodium chloride, P2000 (Dow Chemical), trace elements and lactose or glucose as carbon source. The medium was pH 6.5. Flasks were incubated at 26°C with shaking at 225 rpm. For static 96-well cultures, the same medium was used and the spores were obtained from the purification plate with a wooden toothpick. 96-well plates were incubated, without shaking, at 26°C.

Sampling was done after 5 days for lovastatin. For shake flask experiments 1-1.5 mls of supernatant was placed into 96-well plates, which were centrifuged and supernatants transferred to new 96-well plates. Samples were frozen at -80°C for storage and for later assays.

Cultures that were grown standing in a 96-well plate were centrifuged and the supernatant was transferred to a new 96 well plate. Samples were frozen at -80°C.

PCR analysis of transformants demonstrates that greater than fifty percent of the transformants contain the transgene. Variability in levels of transgene expression can presumably be influenced by integration site and copy number.

**5.4 Measurement of lovastatin production.** Lovastatin concentration was determined by high pressure liquid chromatography (HPLC). Briefly, 100 µL of broth sample was removed and diluted 1:10 into 70% H<sub>2</sub>O-30% acetonitrile (900 µl). This mixture was

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centrifuged to pellet debris at 13000 rpm for 5 minutes. 900 µl of this diluted broth was transferred to a vial and the sample was analyzed by HPLC. 10 µl were injected into a Waters HPLC system (996 photo-diode array detector, 600 E pump controller and 717 autosampler) equipped with a YMC-Pack ODS column (Aq-302-3, 150 x 4.6 mm ID, S-3 µM pore size) and eluted with isocratic 40% aqueous acetic acid (0.7%)-60% acetonitrile for 8 minutes. Lovastatin was detected at 238 nm and was shown to have a retention time of 6.5 minutes. Lovastatin in samples was quantified using a calibration curve created from pure lovastatin samples.

## 6. Example 2

### Regulators of penicillin production in *Penicillium chrysogenum*

6.1 Purpose. The purpose of this experiment was to examine the effect of expressing fungal regulators on secondary metabolite production in *P. chrysogenum*. Improved production of the antibiotic penicillin will aid in the current need for high yielding production processes.

#### 6.2 Methods.

In these experiments we have determined whether overexpressing some of these regulators effects penicillin production by *P. chrysogenum*. Plasmids MB1325 (vector control), MB1310 (*creA*), MB1316 (*lovE*), and MB1317 (*orf13*) were transformed into *P. chrysogenum* strain MF1 (ATCC 9480). *creA* and *lovE* appear in the present specification in Table 1. *orf13* is related to *lovE*; these proteins are zinc finger transcription factors encoded in the lovastatin biosynthetic cluster of *A. terreus*. Transformation of *P. chrysogenum* and measurement of penicillin production were achieved according to the protocols described below. Figure 2, attached to this declaration, is a graphic depiction of penicillin culture concentration, as measured by UV/Spec analysis, from broths of *P. chrysogenum* cultures expressing the regulators. The number of individual transformants tested for each plasmid is listed in parentheses next to the label. Results are shown in standard box plot format. The horizontal line in each individual box represents the median.

The results demonstrate that the three heterologous regulators increase penicillin production in *P. chrysogenum*. Genes for two of these regulators, *lovE* and *orf13* are present in the biosynthetic cluster for another secondary metabolite, lovastatin. This supports the reasoning that regulation of secondary metabolite production occurs through common conserved signaling pathways and thus fungal regulators such as those listed in the present specification are likely to function cross species to regulate secondary metabolite production. Further evidence is provided by the fact that the *A. nidulans creA* gene when overexpressed increases both lovastatin (see Example 1, above) and penicillin production.

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### 6.3 Transformation of *P. chrysogenum*.

Transformation was accomplished in the same manner as described in Example 1 for *A. terreus*, except transformants were selected on 30 µg/mL phleomycin. To test levels of penicillin produced in *P. chrysogenum* transformants, a plug containing spores and mycelia is used as the inoculum. The medium used is the published P2 production medium (Lein (1986), in *Overproduction of Microbial Metabolites*, Vanek and Hostalek (eds.), Butterworth Heinemann, pp. 105-139) that contains 30% lactose, 5X pharmedia cotton seed flour, ammonium sulfate, calcium carbonate, potassium phosphate, potassium sulfate, and phenoxyacetic acid, at pH 7. Flasks were incubated at 26°C with shaking at 225 rpm.

Sampling was done after 6 days of growth. 1-1.5 mls of supernatant were placed into 96-well plates. Plates were centrifuged and supernatants transferred to a new 96-well plate. Standard samples contained 0, 25, 50, 100, 200, 300, 400, and 500 µg/mL phenoxymethylpenicillin (sodium salt) dissolved in 10 mM potassium phosphate (pH 7.0), and assays were conducted as described below.

6.4 Determination of Penicillin Production. Fermentation broth was clarified by centrifugation for 10 minutes at 4000 g, and 40 µL of clarified fermentation broth and penicillin standard solutions was pipetted into individual wells of a 96-well UV collection plate. Next, 200 µL of imidazole reagent was pipetted into a 96-well filter plate (0.45 micron). The derivatization reaction of penicillin was initiated by vacuum filtration of imidazole reagent into a collection plate containing the aliquoted samples and standards. The collection plate was placed into a 96-well plate reader at 45 degrees while absorbance at 325 nm was monitored over 20 minutes. A Molecular Dynamics (Sunnyvale, CA) 96-well UV/Vis plate reader was used for all spectrophotometric detection. A 1.2 M aqueous imidazole solution containing mercuric chloride at a concentration of 1 mM, pH 6.8 was prepared as follows: 8.25 g of imidazole was dissolved in 60 mL of water, 10 mL of 5 M HCl was added, and then 10 mL of a solution of mercuric chloride (0.27 g dissolved in 100 mL of water) was added. The pH was adjusted to 6.80 +/- 0.05 with 5 M HCl and the volume was brought to 100 mL with water (see, e.g., Bundgaard and Ilver (1972), *Journal of Pharm. Pharmac.* 24: 790-794).

## 7. **Example 3**

**The *ganB* G45R dominant active mutant variant increases lovastatin production in *Aspergillus terreus*.**

7.1 Purpose. The purpose of this experiment is to examine the effect of expressing a dominant active mutant variant on secondary metabolite production. Identification of dominant mutant variants that alter secondary metabolite production will be useful in strain improvement strategies.

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## 7.2 Methods.

We introduced a known dominant active mutation into the *A. nidulans* *ganB* gene that encodes a G  $\alpha$  subunit. We overexpressed the *ganB* G45R allele in *A. terreus* in order to observe its effects on lovastatin production. *A. terreus* strain MF22 (ATCC 20542) was transformed with plasmids MB2941(vector control), MB3247 (wild-type *ganB*), and MB3308 (*ganB* G45R). Transformation of *A. terreus* and measurement of lovastatin production were achieved according to the protocols described in Example 1, above. Figure 3, attached to my declaration, is a graphic depiction of lovastatin culture concentration, as measured by HPLC analysis, from broths of *A. terreus* cultures expressing the three regulators. The results from 24 individual transformants for each plasmid are shown in standard box plot format. The horizontal line in each individual box represents the median.

The results demonstrate that *ganB* G45R increases lovastatin levels more than either the vector only control or wild-type *ganB*. The predictability of dominant active and dominant negative mutations among G protein  $\alpha$  subunits indicates that one could test a large number of dominant mutant variants rapidly. Furthermore, the conserved nature of signaling pathways involved in secondary metabolism indicates that, similar to the *A. nidulans* *ganB* G45R variant, other mutant variants will also function cross-species to regulate secondary metabolite production. Fungal homologs of *ganB* are listed in the present specification in Table 1.

## 8. **Example 4**

**The *gna3* G207A dominant negative mutant variant decreases lovastatin production in *Aspergillus terreus*.**

8.1 Purpose. The purpose of this experiment was to examine the effect of expressing a dominant negative mutant variant on secondary metabolite production. Identification of dominant mutant variants that alter secondary metabolite production will be useful in strain improvement strategies. Decreased production of specific (undesired) secondary metabolites is useful for improving purity profiles and/or increasing carbon flow to desired metabolites.

## 8.2 Methods.

We overexpressed the *gna3* G207A allele in *A. terreus* in order to observe its effects on lovastatin production. *A. terreus* strain MF22 (ATCC 20542) was transformed with plasmids MB2941(vector control), MB3252 (wild-type *GNA3*), and MB3320 (*gna3* G207A). Transformation of *A. terreus* and measurement of lovastatin production were achieved according to the protocols described in Example 1. Figure 4, attached to my declaration, is a graphic depiction of lovastatin culture concentration, as measured by HPLC analysis, from broths of *A. terreus* cultures expressing the three regulators. The number of different transformants tested for each plasmid is listed in parentheses next to the label. The horizontal line in each individual box represents the median.

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The results demonstrate that *gna3 G207A* exhibits reduced lovastatin levels when compared to either the vector only control or wild-type *GNA3*. Decreased lovastatin levels would be desired when one wanted to increase production of the secondary metabolite sulochrin. Similar to lovastatin, sulochrin is a polyketide produced by *A. terreus*. Lovastatin would be an undesired metabolite in a sulochrin fermentation because it would shunt carbon atoms away from sulochrin production.

9. **Example 5**  
**Dominant neomorphic variant of An09**  
**increases lovastatin production in *Aspergillus terreus*.**

9.1 **Purpose.** The purpose of this experiment is to examine the effect of expressing a dominant neomorphic mutant variant on secondary metabolite production. Identification of dominant mutant variants that alter secondary metabolite production will be useful in strain improvement strategies.

9.2 **Methods.**

We used the potent VP16 activation domain from herpes simplex virus to create a VP16-An09 fusion construct. We overexpressed this VP16-An09 fusion protein in *A. terreus* and examined its effect on lovastatin production. *A. terreus* strain MF22 (ATCC 20542) was transformed with plasmids MB2143(vector control), MB1617 (*An09*), and MB2203 (*VP16-An09*). Transformation of *A. terreus* and measurement of lovastatin production were achieved according to the protocols described in Example 1. Figure 5, attached to my declaration, is a graphic depiction of lovastatin culture concentration, as measured by HPLC analysis, from broths of *A. terreus* cultures expressing the three regulators. The results from 12 individual transformants for MB2143 and MB1617 and 11 individual transformants for MB2203 are shown in standard box plot format. The horizontal line in each individual box represents the median.

The results demonstrate that VP16-An09 increases lovastatin levels significantly more than the vector control. However, expression of An09 (without the VP16 fusion) does not significantly increase yields of lovastatin. Therefore, fusion of An09 and VP16 confers a new molecule with dominant properties distinct from each of the parents (a dominant neomorph).

10. **Example 6**  
**Conditional expression of modulators**  
**increases lovastatin production in *A. terreus*.**

10.1 **Purpose.** The purpose of this experiment is to conditionally express modulators of secondary metabolite production. Tight regulation of expression patterns of secondary metabolite modulators may be useful in helping dissect which pathways and genes the

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modulators alter, increasing the stability of toxic modulators, and controlling the timing and sequence of metabolite production.

## 10.2 Methods.

In this example we used the strongly inducible xylanase promoters from *A. nidulans* (*xlnA*) and *P. chrysogenum* (*xylP*) genes. The xylanase promoter is repressed in the presence of glucose and induced in the presence of xylose or xylan. *A. terreus* strain MF22 (ATCC 20542) was transformed with either: Experiment A: plasmids MB2941(vector control), MB3428 (*xlnA-Pc23*), and MB3432 (*xlnA-gna3 G44R*) or Experiment B: MB2941(vector control), MB3262(*xlnA-lovE*), and MB3261 (*xlnP-lovE*). The *P. chrysogenum* *Pc23* gene is a putative regulator of fungal gene expression that encodes a GATA zinc finger transcription factor. The *N. crassa* *GNA3* gene encodes a G protein  $\alpha$  subunit. The *gna3 G44R* mutant encodes a dominant active variant that fails to undergo GTP hydrolysis. Fungal homologs of both *Pc23* and *GNA3* are listed in Table 1 of the specification. *lovE*, the transgene present in MB3262 and MB3261, is listed in Table 1 of the specification.

Transformation of *A. terreus* and measurement of lovastatin production were achieved according to the protocols described in Example 1 except production media was either 4% glucose (repressing conditions) or 3% glucose/1% xylose (strong inducing conditions as determined empirically). Figure 6, attached to my declaration, is a graphic depiction of lovastatin culture concentration, as measured by HPLC analysis, from broths of *A. terreus* cultures expressing the regulators. The results from 12 individual transformants for each plasmid in Experiment A and 10 individual transformants for each plasmid in Experiment B are shown in standard box plot format. The horizontal line in each individual box represents the median.

The results demonstrate increased levels of lovastatin are observed in the presence of inducing conditions (blue) compared to repressing conditions (red), except in the presence of control, vector-only plasmids. In some cases it appears that a slight increase in lovastatin levels may also be apparent in repressing conditions (red) when compared to the vector controls. It is possible that under conditions of carbon starvation (i.e., all the glucose has been utilized) derepression of the *xlnA* and *xylP* promoters may occur. This phenomenon has not been investigated further.

In conclusion, we have conditionally overexpressed one dominant mutant variant and two different wild-type modulators of secondary metabolite production using two different regulated promoters. In each case we observed conditional, increased lovastatin production.



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11. **Example 7**  
**Small molecule modulators of secondary metabolite production**

11.1 **Purpose.** The purpose of this experiment was to examine the effect of small molecule modulators on secondary metabolite production. Identification of small molecule modulators of secondary metabolite production will be extremely useful in strain improvement strategies.

11.2 **Methods.**

We examined the effect of small molecule modulators on red pigment production in *Monascus*. Fungal strains MF32 (ATCC 20657-*Monascus ruber*) and MF148 (ATCC 16435-*Monascus spp.*) were used in these experiments. The protocol was as follows: Inoculate spores onto Hiroi agar (2% agar, 10% sucrose, 1% casamino acids, 0.6% yeast extract, 0.2% NaNO<sub>3</sub>, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.05% KCl, 0.0015% FeSO<sub>4</sub>•7H<sub>2</sub>O). Use 10<sup>4</sup> spores for a 12-well plate (containing 2 ml agar/well), and 10<sup>3</sup> spores for a 96 well plate (containing 0.15 ml agar/well). Incubate overnight at 30°C. Add small molecule modulator. Incubate 1-3 additional days at 30°C. The protein synthesis inhibitor, cycloheximide (Chx) (MW 281.4), and the HMG-CoA reductase inhibitor, lovastatin (Lov) (MW 422.255) were tested for effects on red pigment production. Water was used as a diluent for the cycloheximide and as a control. Lovastatin was diluted in 0.2 M Tris-HCl (in data not shown, 0.2 M Tris-HCl was observed to have no effect on red pigment production).

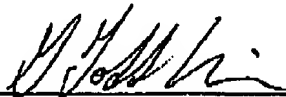
Figure 7, attached to my declaration, shows that addition of low and high concentrations of both cycloheximide and lovastatin increase red pigment production in MF148. In contrast, only addition of high concentrations of cycloheximide increase red pigment production in MF32. This simple colorimetric assay is readily adaptable to high throughput analysis. The wide availability of small molecule libraries combined with the automatable assay we describe in these experiments suggests that one could readily identify numerous small molecule modulators of secondary metabolite production.

12. The data presented in these examples clearly demonstrate that one of skill in the art can follow the teachings of the specification as a guide to achieve the invention claimed in claims 1-28 and 102 and 103.

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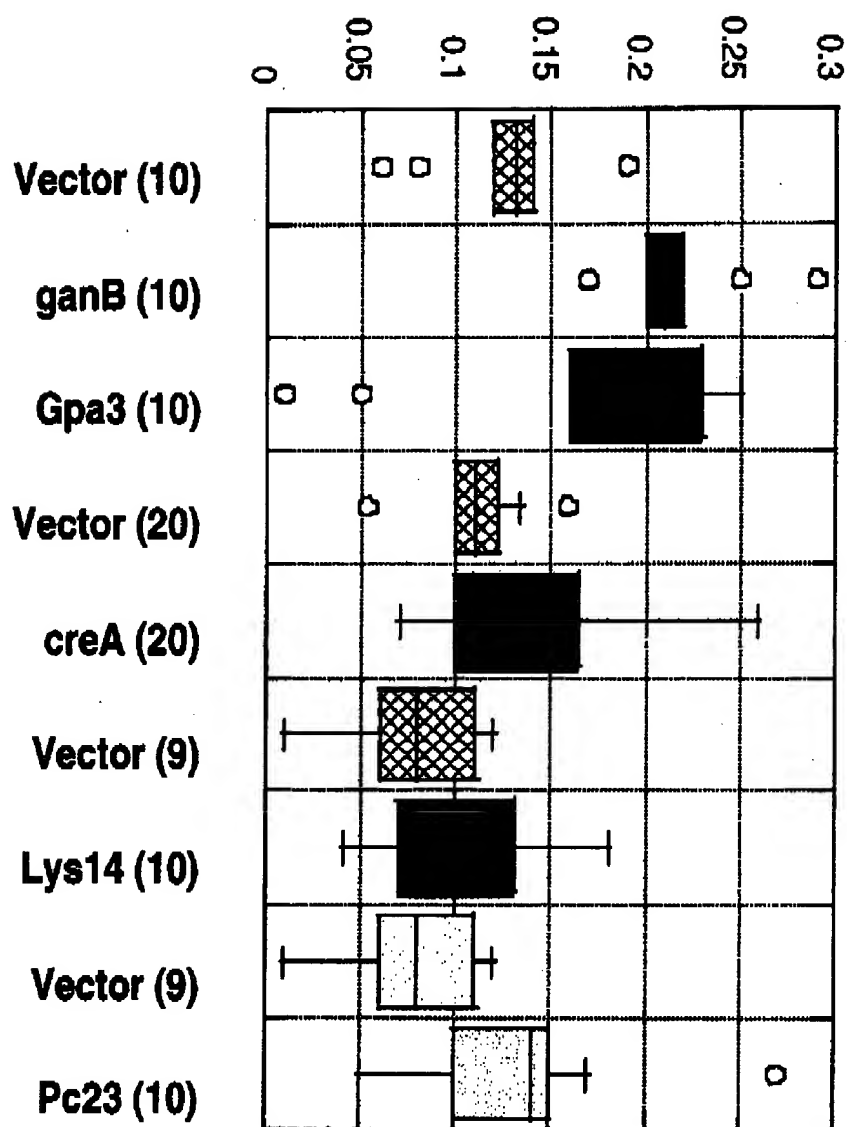
13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

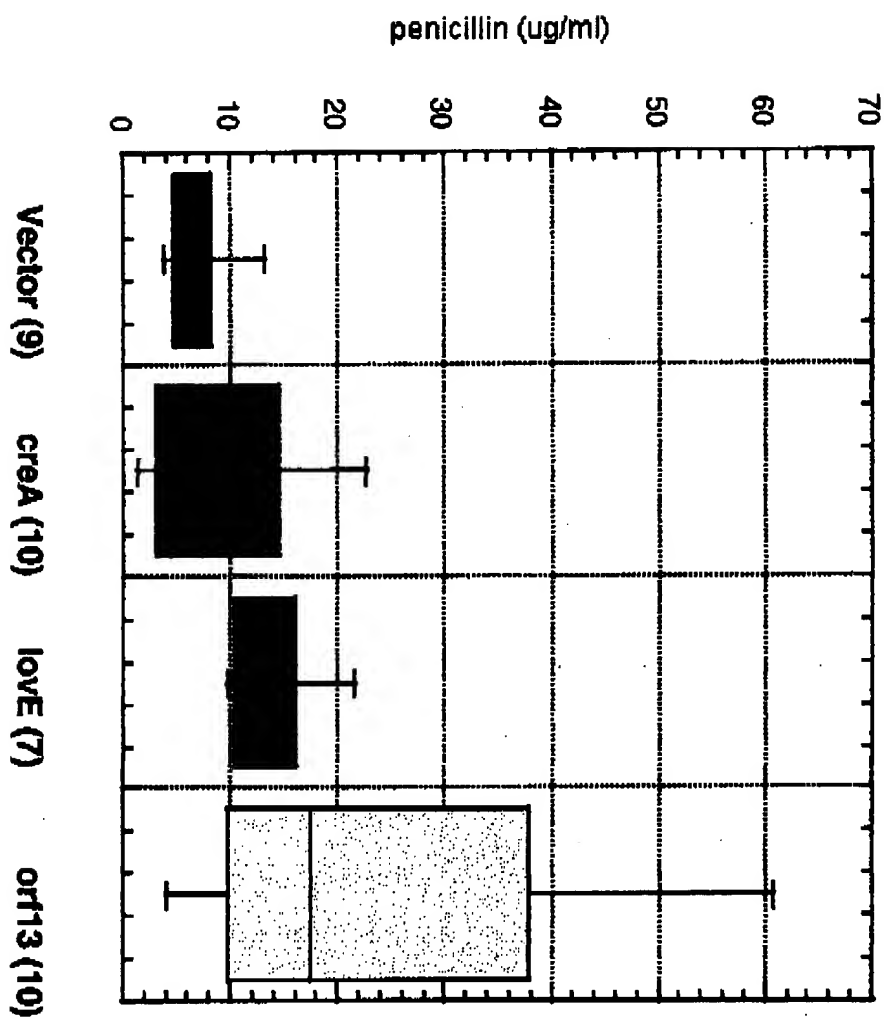
Date:

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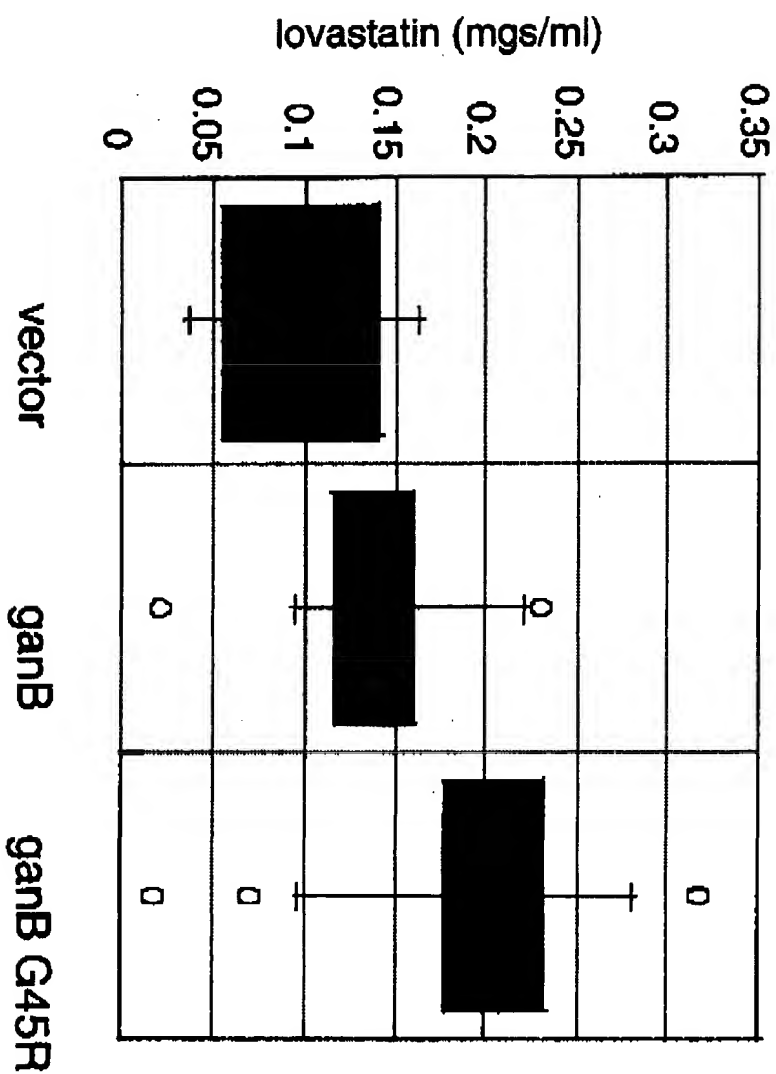
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**Figure 1. Regulators of lovastatin production in *A. terreus***

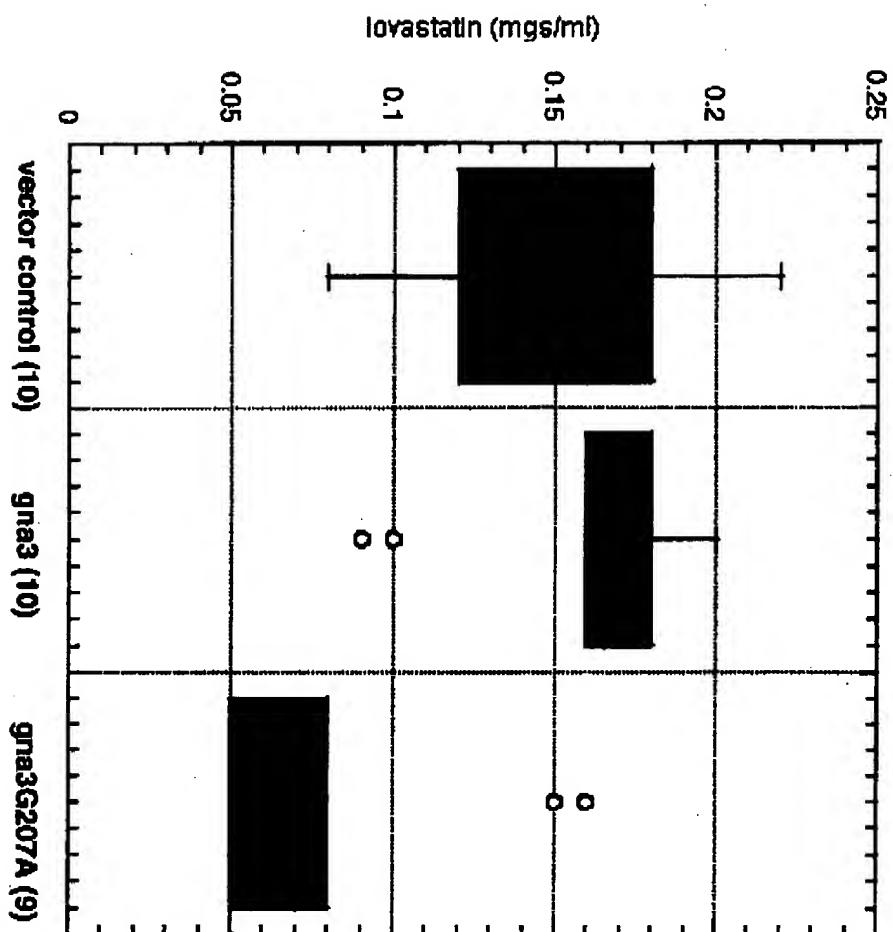


**Figure 2. Regulators of penicillin production in *P. chrysogenum***

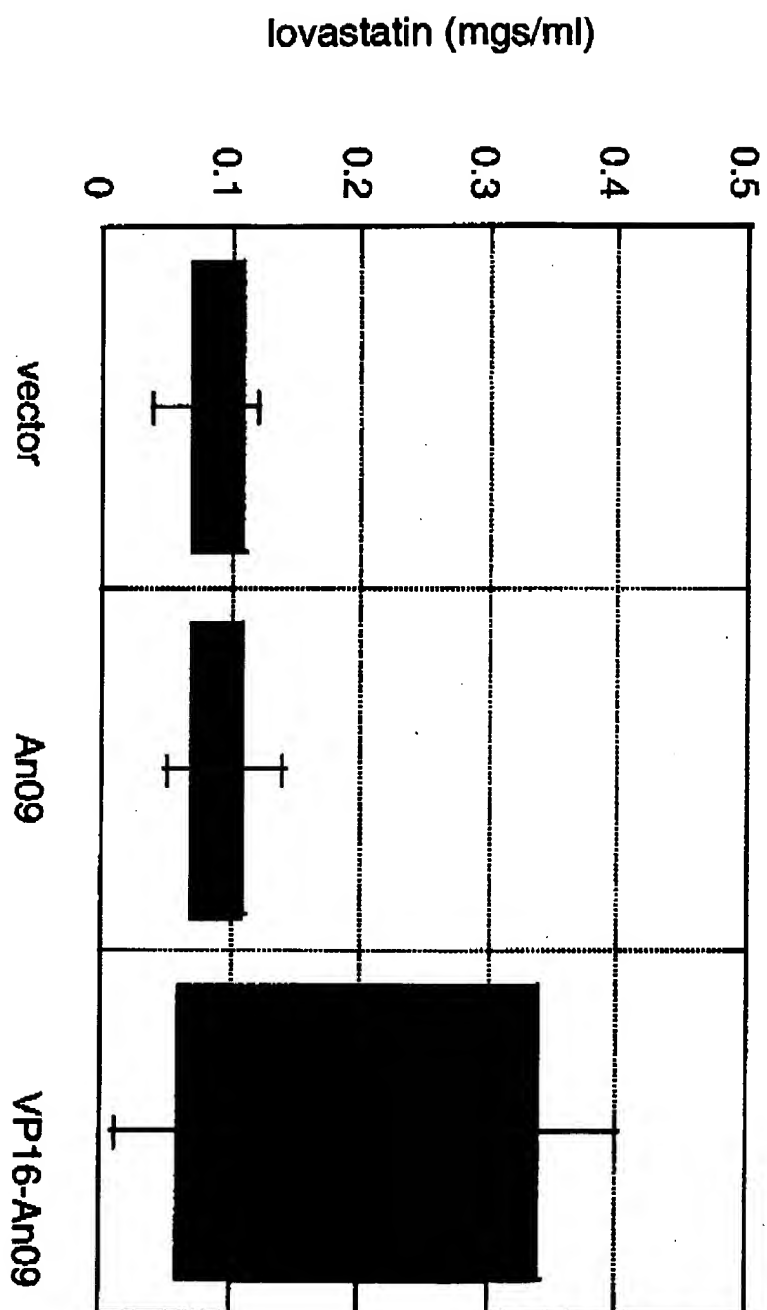
**Figure 3. Dominant active ganB G45R variant increases lovastatin levels in *A. terreus***



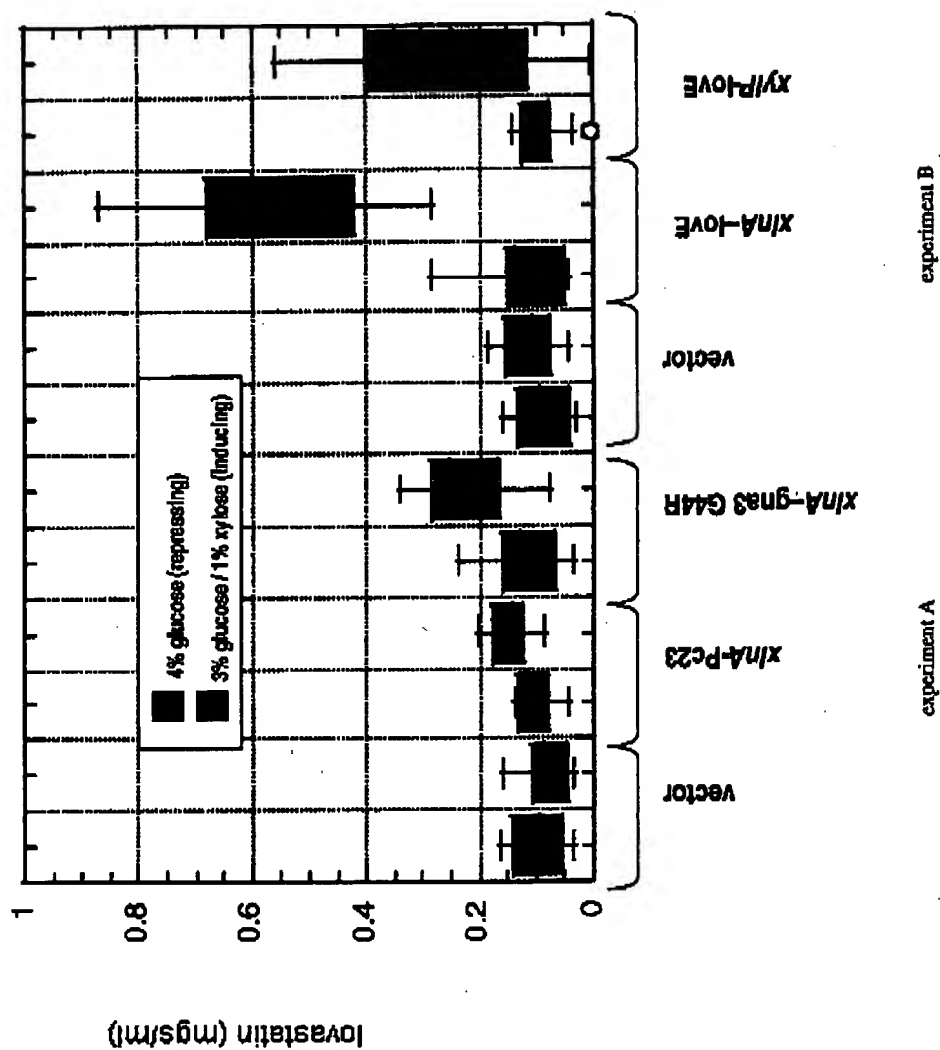
**Figure 4. Dominant negative gna3 G207A variant decreases lovastatin levels in *A. terreus***



**Figure 5. Dominant neomorphic VP16-An09 increases lovastatin levels in *A. terreus***

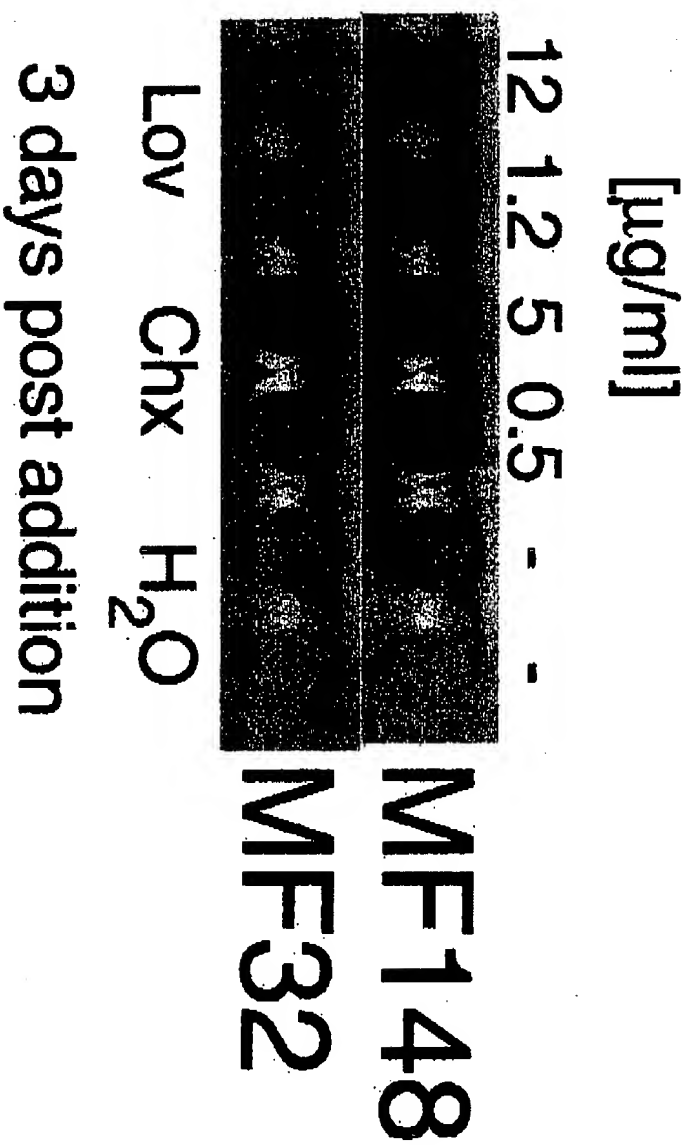


**Figure 6. Conditional expression of modulators increases lovastatin production in *A. terreus***





## Figure 7. Small molecule modulators of secondary metabolite production



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- 1989-1995 Graduate student in the laboratory of Dr. David Weaver  
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- 1988 Summer Research Assistant in the laboratory of Dr. D. Liston, Dept. of Neuroscience and Cancer Research  
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1990 Honorable Mention, NSF Graduate Fellowship  
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**PUBLICATIONS**

- Milne, G.T. and D.T. Weaver (1993). Dominant negative alleles of *RAD52* reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes & Development*. 7(9):1755-65.
- Donovan, J.W., G.T. Milne, and D.T. Weaver (1994). Homotypic and heterotypic protein associations control Rad51 function in double-strand break repair. *Genes & Development*. 8(21):2552-2562.
- Milne, G.T., T. Ho, and D.T. Weaver (1995). Modulation of *Saccharomyces cerevisiae* DNA double-strand break repair by Srs2 and Rad51. *Genetics*. 139(3):1189-99.
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- Milne, G.T., J.W. Donovan, Y. Xiao, and D.T. Weaver (1996). "Dominant negative mutations and comparative gene analysis in DNA repair" in *Microbial Genome Methods*. Academic Press.